PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
Gang Chen, et al.

Serial No.: 09/699,023

Filed: October 27, 2000

For: ISOLATION OF BINDING PROTEINS WITH HIGH AFFINITY TO LIGANDS

Group Art Unit: 1645

Examiner: Ford, V.

Atty. Dkt. No.: UTSB:675US

CERTIFICATE OF MAILING
37 C.F.R. §1.8

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Robert E. Hanson

BRIEF ON APPEAL

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BRIEF ON APPEAL

Mail Stop Appeal Brief - Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

Appellants hereby submit an original and two copies of this Appeal Brief in response to the Final Office Action dated March 4, 2004. This Brief is filed pursuant to the Notice of Appeal mailed September 7, 2004. The due date for the Brief is December 13, 2004 in view of the enclosed Petition for Extension of Time and receipt of the Notice of Appeal by the Office on September 13, 2004.

The extension fee and fee for filing this Appeal Brief are attached. No additional fees are believed due in connection with this paper. However, should any other fees be due, or the attached fee be deficient or absent, the Commissioner is authorized to withdraw the appropriate fee from Fulbright & Jaworski L.L.P. Deposit Account No. 50-1212/UTSB:675.

Please date stamp and return the enclosed postcard to evidence receipt of this document.

PETITION FOR EXTENSION OF TIME

Pursuant to 37 C.F.R. § 1.136(a), Appellants petitions for an extension of time of one month to and including December 13, 2004, in which to file this Appeal Brief. Pursuant to 37 C.F.R. § 1.17, a check is enclosed including the process fee of $60.00 for a one-month extension of time. If the check is inadvertently omitted, or should any additional fees under 37 C.F.R. §§ 1.16 to 1.21 be required for any reason relating to the enclosed materials, or should an overpayment be included herein, the Commissioner is authorized to deduct or credit said fees from or to Fulbright & Jaworski Deposit Account No. 50-1212/UTSB:675US

I. REAL PARTY IN INTEREST

The real party in interest is the assignee, The Board Of Regents, The University Of Texas System.

II. RELATED APPEALS AND INTERFERENCES

There are no interferences or appeals for related cases.

III. STATUS OF THE CLAIMS

Claims 1-74 were initially filed. Claims 33-74 were withdrawn from consideration as directed to non-elected subject matter. Claims 1-74 were pending at the time of the final Office Action dated March 4, 2004 and claims 1-32 were rejected. Claim 3 was canceled in an Amendment and Response to Fourth Office Action filed by Appellants on May 4, 2004. It is unknown if the Amendment has been entered.

Prior to entry of the Amendment claims 1-74 were pending and after entry of the amendment claims 1-2 and 4-74 will be pending. Copies of the pending claims with and without entry of the Amendment are attached as Appendix 1 and Appendix 2, respectively.

IV. STATUS OF AMENDMENTS

A claim amendment was filed with the Amendment and Response to Fourth Office Action dated May 4, 2004 that amended claim 1 and canceled claim 3. The status of the Amendment is unknown.

V. SUMMARY OF CLAIMED SUBJECT MATTER

The invention relates to a method of obtaining a bacterium comprising a nucleic acid sequence encoding a binding protein capable of binding a target ligand. Specification at p.6, l.10-12. In one embodiment the method comprises the steps of: (a) providing a Gram negative bacterium comprising a nucleic acid sequence encoding a candidate binding protein, wherein said binding protein is expressed in soluble form in the periplasm of said bacterium; (b) contacting said bacterium with a labeled ligand capable of diffusing into said bacterium periplasm; and (c) selecting said bacterium based on the presence of said labeled ligand within the periplasm. Specification at p.6, l.12-17. The method may further comprise obtaining a nucleic acid sequence encoding a binding protein capable of binding a target ligand by cloning said nucleic acid sequence encoding said candidate binding protein. Specification at p.6, l.18-26.

VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

A. Are claims 1-32 lacking enablement under 35 U.S.C. § 112, first paragraph?

B. Are claims 1-32 indefinite under 35 U.S.C. § 112, second paragraph?

VII. ARGUMENT

A. The Claims Are Enabled Under 35 U.S.C. §112, First Paragraph

The Examiner maintains the rejection of claims 1-32 under 35 U.S.C. §112, first paragraph, as not being enabled. In particular, the Examiner presents three main points of alleged non-enablement: (1) it is stated that the claims are not enabled in the absence of a wash step, (2) it is stated that enablement is not provided for use of ligands greater than 2000 Da, and (3) it is alleged that use of the claimed method with a nucleic acid as the labeled ligand is not

enabled. It is additionally stated that use of enzymes generally with the invention is not enabled. The rejection should be reversed by the Board as set forth below.

(1) Appellants have demonstrated that a wash step is not necessary

Appellants note that the addition of a wash step is not required for use of the invention as alleged by the Examiner. In support of this, Appellants provided during prosecution the Declaration of Jongsik Gam. **Appendix 3, Exhibit A**. As described by Mr. Gam, the claimed methods have been used both with and without the addition of a wash step to demonstrate that the wash step is not necessary. It was demonstrated in particular that cells expressing a binding protein having affinity for a labeled ligand can be detected away from control cells based on the presence of the labeled ligand bound in the periplasm with or without a wash step. Mr. Gam explains that the specific interaction of the binding protein and labeled ligand in the periplasm of the bacterium ***retains*** and ***concentrates*** the labeled ligand inside the periplasm of ***only*** those cells with high affinity binding proteins. The concentration of the labeled ligand bound to the binding protein in the periplasm makes the cells detectable regardless of the presence of unbound labeled ligand. *Id.*

As Appellants have demonstrated that this additional step is not required, and the claimed method is fully enabled without the step, reversal of the rejection is respectfully requested.

(2) The invention is not properly limited to ligands of less than 2000 Da or specific classes of ligands

The Examiner continues to reject the claims based on the allegation that labeled ligands of more than 2000 Da are not "capable of diffusing" into a cell. The evidence Appellants provided in the previous response included a peer reviewed publication by Chen *et al.*, (**Appendix 3, Exhibit B**) showing that oligonucleotide 20mers (8,727 Da) enter the bacterial

cell. However, the Examiner asserted that this was unpersuasive based on the allegation that facilitated transport is required for molecules of such size to enter the cell.

(a) Interpretation of the claims

Appellants initially note that the maintenance of the rejection appears to be based on confusion regarding the meaning of the term "capable of diffusing" as used in claim 1. The Examiner has cited a definition from Webster's for the term "diffusion" which is said to be limited to spontaneous (*e.g.*, unfacilitated) movement. However, the claims are not so limited. The definition given is but one of several library definitions available for terms that are related to the actual original claim term "capable of diffusing into said bacterium." Neither the other definitions nor the specification are limited in this way.

The relevant definition of "diffuse," which has the inflected form "diffusing," from the Merriam-Webster™ Online dictionary is "to spread out or *become transmitted especially by contact.*" **Appendix 3, Exhibit C** (emphasis added). Similarly, the Cambridge Online Dictionary™ (http://dictionary.cambridge.org/) defined "diffuse" as a verb to mean "1 to (cause something to) spread in many directions" or "(2) to (cause a gas or liquid to) *spread through or into a surrounding substance by mixing with it.*" **Appendix 3, Exhibit D** (emphasis added).

Consistent with the foregoing, nothing in the specification requires that labeled ligands enter the bacterium by any given mechanism. Therefore, neither the art known meaning of the claim terms nor the specification are consistent with the position taken by the Examiner regarding the interpretation of "diffusing." Even assuming this to be true for the purpose of argument, although it is not, this at best goes to "how" or "why" the invention works, which is irrelevant under the first paragraph of 35 U.S.C. §112, first paragraph. All that is relevant under this section is that one of skill in the art can make and use the claimed invention without undue experimentation, which has fully been demonstrated herein.

(b) Chen *et al.* demonstrates the enablement of the claims

Enablement speaks to the ability to make and use the claimed invention. The mechanisms by which ligands enter the cell is irrelevant to the function of the technique. Here, Appellants have submitted a peer-reviewed article, Chen *et al.*, that specifically shows that ligands of at least 10kDa can enter the periplasmic space. **Appendix 3, Exhibit B**. Regardless of the molecular mechanisms involved, this alone provides clear evidence for enablement of ligands of at least the 10 kDa acknowledged by the Examiner.

The Examiner states that the Chen *et al.* reference is not persuasive because "proper conditions" are required such as treatments with filamentous bacteriophages or growth under sub-optimal conditions. However, the specification ***specifically teaches*** these techniques as well as many other such techniques for permeabilization of bacteria. For example, the use of ***filamentous bacteriophages*** to increase permeability is described in the last paragraph of ***page 16*** of the specification. The use of ***sub-optimal temperatures*** is specifically taught in the first paragraph of ***Example 3*** of the specification. The specification further describes numerous other techniques that were known in the art for increasing bacterial permeability. For example, Fig. 8 shows that an increase in FACS signal was obtained when cells expressing periplasmic scFv antibodies to digoxigenin were labeled with this probe using 5X PBS to permeabilize the outer membrane. Therefore, given that the "proper conditions" for use of labeled ligands of at least 10 kDa are both fully described in the specification and known in the art, there is no basis whatsoever to conclude that Appellants' claims are not fully enabled.

(c) The rejection was not applied with respect to the claimed invention

Appellants finally note that the rejection appears to have not been applied with respect to the claimed invention. All that is required under 35 U.S.C. §112, first paragraph, is that the specification teach one reasonably skilled in the art how to make and use ***what is claimed***

without undue experimentation. *In re Wands*, 858 F.2d 731, 737, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988); *see also Durel Corp. v. Osram Sylvania Inc.*, 256 F.3d 1298, 1306-07 (Fed. Cir. 2001). The focus of examination must therefore be whether the subject matter within the scope of the claims is enabled. M.P.E.P. § 2164.08.

Here, step (b) of current claim 1 comprises contacting a bacterium with a labeled ligand "capable of diffusing" into the periplasm of the bacterium. To the extent that a given ligand is not capable of diffusing into the periplasm as alleged by the Examiner, the ligand is not within the scope of the claims. Therefore, even assuming *arguendo* that the allegations made by the Examiner are correct, which they are not, the full scope of the claims is enabled. *Id.*

In view of the foregoing, reversal of the rejection is respectfully requested.

(3) The use of nucleic acids has been enabled

The Examiner continues to allege that enablement has not been provided for use of nucleic acids as ligands. In response it is first noted that the comments of the Examiner regarding the means by which nucleic acids enter the cell are irrelevant given that the claims do not require that a labeled ligand enter the periplasm by any particular method. What is relevant is that the studies carried out by the inventors and working examples in the specification demonstrate enablement for use of labeled ligands comprising nucleic acids. For example, the Chen *et al.* (2001) reference (**Appendix 3, Exhibit B**) describes the use of labeled ligands comprising nucleic acids for detection in the periplasm of *E. coli* bacteria expressing a binding protein. In particular, oligonucleotide 10mers (4,897 Da) and even 20mers (8,727 Da) were labeled with digoxigenin and a fluorescent label, resulting in the successful labeling of *E. coli* in 5X PBS expressing scFv antibodies binding the digoxin in the periplasm. (see Fig. 3, page 539). The studies demonstrate enablement for use of nucleic acids.

Use of labeled ligands comprising nucleic acids is also described in Example 8 of the specification. This example demonstrates that a fluorescently-tagged oligonucleotide (2384 Da molecular weight) diffused into bacteria, and specific ligand-binding protein binding occurred. No significant DNA-labeled probe background signal was observed in Example 8, and, because no evidence exists that this kind of interaction would present a significant problem for use of the invention, the idea suggested by the Examiner that DNA-labeled probe binding will prevent use of nucleic acids as ligands must be regarded as pure speculation. The nucleic acids that are used as ligands can be easily distinguished from other nucleic acids because the ligand nucleic acids are tagged with a fluorescent (or other) labeling moiety.

The studies demonstrate that nucleic acids can enter the periplasm. This further demonstrates that entry of labeled ligands into the periplasm is not limited to only certain types of molecules of very limited size. In this case, the nucleic acid is not used as a source of genetic information to be transcribed and translated into protein. Rather, the nucleic acid is being used as a scaffold to contain the ligand of the binding protein as well as a fluorescent label. Therefore, again, non-specific binding with native nucleic acids does not affect the ability to identify selectively bound labeled ligand.

(4) Use of enzymes is enabled

The Examiner continues to assert that the specification does not enable use of enzymes generally. In particular, the Examiner asserts that, although use of *Fusarium solani* lipase cutinase is described in the specification, the specification has not shown that other enzymes and substrates diffuse across the bacterial outer membrane. It is stated that different substrates behave differently and require different transport systems to cross the outer membrane.

However, Appellants have already shown that the claims do not require that a labeled ligand enter the periplasm by any specific method. All that is relevant is that the specification

teaches how to use enzymes in connection with the invention as claimed, which it does. For example, the membrane permeabilization methods described by Appellants are non-specific. This is supported by the multiple different types of labeled ligands that were used and shown to successfully enter the periplasm, including nucleic acids, oligopeptides and a polyethylene glycol derivative, including substrate for *Fusarium solani* lipase cutinase. There is no basis to conclude that only certain types of substrates will cross the outer membrane.

No basis has also been provided to conclude why the *Fusarium solani* lipase cutinase example is not representative of the claims in general. This constitutes a working example within the scope of the claims. Given the demonstrated ability to introduce a wide variety of molecules of different sizes into the *E. coli* periplasm, there is no basis to conclude that the example does not enable the full scope of the claims. Any suggestions to the contrary are unsupported speculation given the evidence presented by Appellants.

(5) The rejection must be reversed under the APA

Appellants have established above that the Examiner failed to provide evidence supporting the rejection and instead has disregarded evidence presented by Appellants affirmatively establishing enablement in favor of personal opinion and conjecture. However, it is specifically the burden of the Examiner to provide such evidence to support of the rejections made and to consider the evidence presented. Such evidence may not be merely disregarded without a firm evidentiary basis for doing so. *In re Dinh-Nguyen*, 181 U.S.P.Q. 46 (CCPA 1974). This showing is further required under the Administrative Procedure Act ("APA"), which establishes that findings of fact and conclusions of law by the U.S. Patent and Trademark Office must be supported by "substantial evidence" within the record in accordance with the 5 U.S.C. § 706(A), (E). *See Dickinson v. Zurko,* 527 U.S. 150, 50 USPQ2d 1930 (1999), *see also In re Gartside*, 203 F.3d 1305, 53 USPQ2d 1769 (Fed. Cir. 2000). The current rejections have not

been supported in fact or law. The standards of the APA have therefore not been met and thus the rejection must be reversed.

In view of the foregoing, Appellants respectfully request that the Board reverse the rejection of claims 1-32 under 35 U.S.C. § 112, first paragraph.

B. The Claims Are Not Indefinite Under 35 U.S.C. §112, Second Paragraph

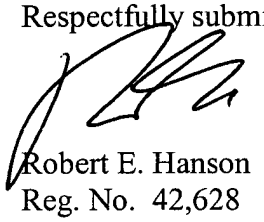
The Examiner rejects claims 1-32 under 35 U.S.C. §112, second paragraph, as being incomplete for the omission of a wash step. However, as demonstrated in the Declaration of Mr. Jongsik Gam (**Appendix 3, Exhibit A**), the addition of a wash step is not required for the function of the invention and therefore this element need not be recited in the claims. Specifically, Mr. Gam explains that the claimed methods have been used both with and without the addition of a wash step, the results of which demonstrate that the wash step is not necessary. It was demonstrated in particular that cells expressing a binding protein having affinity for a labeled ligand can be detected away from control cells based on the presence of the labeled ligand bound in the periplasm with or without a wash step. Mr. Gam explains that the specific interaction of the binding protein and labeled ligand in the periplasm of the bacterium ***retains*** and ***concentrates*** the labeled ligand inside the periplasm of ***only*** those cells with high affinity binding proteins. The concentration of the labeled ligand bound to the binding protein in the periplasm makes the cells detectable regardless of the presence of unbound labeled ligand. Mr. Gam therefore concludes by stating that, "[b]ased on the foregoing studies, a wash step is not required for the use of the method described in the claims of this patent application to obtain a bacterium comprising a nucleic acid sequence encoding a binding protein capable of binding a target ligand."

The Examiner has provided absolutely no basis to doubt the foregoing evidence, which demonstrates that the cited wash step is not essential. It is black-letter law that an applicant need only recite those elements that are essential to the function of the claimed invention. Non-essential embodiments from the disclosure need not be included in the claims. Reversal of the rejection is thus respectfully requested.

VIII. CONCLUSION

It is respectfully submitted, in light of the above, that none of the pending claims are properly rejected under 35 U.S.C. §112, first or second paragraph. Reversal of the pending grounds for rejection is thus respectfully requested.

Respectfully submitted,



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Date: December 13, 2004

APPENDIX 1: LISTING OF APPEALED CLAIMS PRIOR TO ENTRY OF THE AMENDMENT AND RESPONSE TO FOURTH OFFICE ACTION DATED MAY 4, 2004

1. (Previously presented) A method of obtaining a bacterium comprising a nucleic acid sequence encoding a binding protein capable of binding a target ligand comprising the steps of:

(a) providing a Gram negative bacterium comprising a nucleic acid sequence encoding a candidate binding protein, wherein said binding protein is expressed in soluble form in said bacterium;

(b) contacting said bacterium with a labeled ligand capable of diffusing into said bacterium; and

(c) selecting said bacterium based on the presence of said labeled ligand within the bacterium, wherein said ligand and said candidate binding protein are bound in said bacterium.

2. (Original) The method of claim 1, further defined as a method of obtaining a nucleic acid sequence encoding a binding protein capable of binding a target ligand, the method further comprising the step of:

(d) cloning said nucleic acid sequence encoding said candidate binding protein.

3. (Original) The method of claim 1, wherein said binding protein is expressed in soluble form in the periplasm of said bacterium.

4. (Original) The method of claim 3, wherein said nucleic acid sequence encoding a candidate binding protein is further defined as operably linked to a leader sequence capable of directing expression of said candidate binding protein in said periplasm.

5. (Original) The method of claim 1, wherein said Gram negative bacterium is an *E. coli* bacterium.

6. (Original) The method of claim 1, further defined as comprising providing a population of Gram negative bacteria.

7. (Original) The method of claim 6, wherein said population of bacteria is further defined as collectively capable of expressing a plurality of candidate binding proteins.

8. (Original) The method of claim 7, wherein said population of bacteria is obtained by a method comprising the steps of:

(a) preparing a plurality DNA inserts which collectively encode a plurality of different potential binding proteins, and

(b) transforming a population of Gram negative bacteria with said DNA inserts.

9. (Original) The method of claim 6, wherein said population of Gram negative bacteria is contacted with said labeled ligand.

10. (Original) The method of claim 1, wherein said candidate binding protein is further defined as an antibody or fragment thereof.

11. (Original) The method of claim 1, wherein said candidate binding protein is further defined as a binding protein other than an antibody.

12. (Original) The method of claim 1, wherein said candidate binding protein is further defined as an enzyme.

13. (Original) The method of claim 1, wherein said candidate binding protein is further defined as not capable of diffusing out of said periplasm in intact bacteria.

14. (Original) The method of claim 1, wherein said labeled ligand comprises a peptide.

15. (Original) The method of claim 1, wherein said labeled ligand comprises a polypeptide.

16. (Original) The method of claim 1, wherein said labeled ligand comprises an enzyme.

17. (Original) The method of claim 1 where said labeled ligand comprises a nucleic acid.

18. (Original) The method of claim 1, wherein said labeled ligand is further defined as comprising a molecular weight of less than about 20,000 Da.

19. (Original) The method of claim 1, wherein said labeled ligand is further defined as comprising a molecular weight of less than about 5,000 Da.

20. (Original) The method of claim 1, wherein said labeled ligand is further defined as comprising a molecular weight of greater than 600 Da and less than about 30,000 Da.

21. (Original) The method of claim 1, wherein said labeled ligand is further defined as fluorescently labeled.

22. (Previously presented) The methods of claim 1, wherein said nucleic acid encoding a candidate binding protein is further defined as capable of being amplified following said selection.

23. (Original) The method of claim 1, further comprising treating said bacterium to facilitate said diffusing into said periplasm.

24. (Original) The method of claim 23, comprising treating the bacterium with hyperosmotic conditions.

25. (Original) The method of claim 23, comprising treating the bacterium with physical stress.

26. (Original) The method of claim 24, comprising treating the bacterium with a phage.

27. (Original) The method of claim 1, wherein said bacterium is grown at a sub-physiological temperature.

28. (Original) The method of claim 27, wherein said sub-physiological temperature is about 25°C

29. (Original) The method of claim 1, further comprising removing labeled ligand not bound to said candidate binding protein.

30. (Previously presented) The method of claim 1, wherein said selecting comprises fluorescent activated cell sorting.

31. (Original) The method of claim 1, wherein said selecting comprises magnetic separation.

32. (Original) The method of claim 1, wherein said ligand and said candidate binding protein are reversibly bound in said periplasm.

33. (Withdrawn) A method of obtaining a bacterium comprising a nucleic acid sequence encoding a catalytic protein catalyzing a chemical reaction involving a target substrate, the method comprising the steps of:

(a) providing a Gram negative bacterium comprising a nucleic acid sequence encoding a candidate catalytic protein, wherein said catalytic protein is expressed in soluble form in said bacterium;

(b) contacting said bacterium with a target substrate capable of diffusing into said bacterium, wherein said candidate catalytic protein catalyzes a chemical reaction involving said target substrate and wherein said chemical reaction yields at least a first substrate product; and

(c) selecting said bacterium based on the presence of said first substrate product.

34. (Withdrawn) The method of claim 33, further defined as a method of obtaining a nucleic acid sequence encoding a catalytic protein catalyzing a reaction with a target substrate, the method further comprising the step of:

(d) cloning said nucleic acid sequence encoding said candidate catalytic protein.

35. (Withdrawn) The method of claim 33, wherein said candidate catalytic protein is expressed in soluble form in the periplasm of said bacterium.

36. (Withdrawn) The method of claim 35, wherein said nucleic acid sequence encoding a candidate catalytic protein is further defined as operably linked to a leader sequence capable of directing expression of said candidate catalytic protein in said periplasm.

37. (Withdrawn) The method of claim 33, wherein said Gram negative bacterium is an *E. coli* bacterium.

38. (Withdrawn) The method of claim 33, further defined as comprising providing a population of Gram negative bacteria.

39. (Withdrawn) The method of claim 38, wherein said population of bacteria is further defined as collectively capable of expressing a plurality of candidate catalytic proteins.

40. (Withdrawn) The method of claim 39, wherein said population of bacteria is obtained by a method comprising the steps of:

(a) preparing a plurality DNA inserts which collectively encode a plurality of different candidate catalytic proteins, and

(b) transforming a population of Gram negative bacteria with said DNA inserts.

41. (Withdrawn) The method of claim 38, wherein said population of Gram negative bacteria is contacted with said target substrate.

42. (Withdrawn) The method of claim 33, wherein said candidate catalytic protein is further defined as an enzyme.

43. (Withdrawn) The method of claim 33, wherein said candidate catalytic protein is further defined as not capable of diffusing out of said periplasm.

44. (Withdrawn) The method of claim 33, wherein said target substrate comprises a molecule containing a scissile amide bond.

45. (Withdrawn) The method of claim 33, wherein said target substrate comprises a polypeptide.

46. (Withdrawn) The method of claim 33, wherein said target substrate comprises a molecule containing a scissile carboxylic ester bond.

47. (Withdrawn) The method of claim 33, wherein said target substrate comprises a nucleic acid.

48. (Withdrawn) The method of claim 33, wherein said target substrate comprises a molecule containing a scissile phosphate ester bond.

49. (Withdrawn) The method of claim 33, wherein said target substrate comprises a molecule containing a scissile sulfonate ester bond.

50. (Withdrawn) The method of claim 33, wherein said target substrate comprises a molecule containing a scissile carbonate ester bond.

51. (Withdrawn) The method of claim 33, wherein said target substrate comprises a molecule containing a scissile carbamate bond.

52. (Withdrawn) The method of claim 33, wherein said target substrate comprises a molecule containing a scissile thioester bond.

53. (Withdrawn) The method of claim 33, wherein said target substrate is further defined as comprising a molecular weight of less than about 20,000 Da.

54. (Withdrawn) The method of claim 33, wherein said target substrate is further defined as comprising a molecular weight of less than about 5,000 Da.

55. (Withdrawn) The method of claim 33, wherein said target substrate is further defined as comprising a molecular weight of less than about 3,000 Da.

56. (Withdrawn) The method of claim 33, wherein said target substrate is further defined as comprising a molecular weight of greater than about 600 Da and less than about 30,000 Da.

57. (Withdrawn) The method of claim 33, wherein said first substrate product is further defined as capable of being detected based on the presence of a fluorescent signature.

58. The method of claim 57, wherein said fluorescent signature is absent in said target substrate.

59. (Withdrawn) The method of claim 58, wherein said fluorescent signature is produced by catalytic cleavage of a scissile bond.

60. (Withdrawn) The method of claim 59, further defined as comprising use of a FRET system, said FRET system comprising a fluorophore bound by a scissile bond to at least a first molecule capable of quenching the fluorescence of said fluorophore, wherein cleavage of said scissile bond allows said first molecule to diffuse away from the fluorophore and wherein the fluorescence of said fluorophore becomes detectable.

61. (Withdrawn) The method of claim 60, wherein the fluorophore comprises a positive charge allowing the fluorophore to remain associated with the bacterium.

62. The method of claim 57, wherein said target substrate is further defined as comprising a latent fluorescent moiety capable of being released by said chemical reaction involving said target substrate.

63. (Withdrawn) The method of claim 62, wherein the latent fluorescent moiety released by said cleavage possesses an overall positive charge allowing said moiety to remain associated with the bacterium following said cleavage.

64. (Withdrawn) The method of claim 57, further defined as comprising labeling said target substrate with a fluorescent pH probe capable of being detected upon a change in pH associated with said chemical reaction involving said target substrate.

65. (Withdrawn) The method of claim 64, wherein said fluorescent pH probe possesses an overall positive charge allowing said fluorescent pH probe to remain associated with the bacterium following said chemical reaction involving said target substrate.

66. (Withdrawn) The method of claim 33, wherein said bacterium is further defined as viable following said selecting.

67. (Withdrawn) The method of claim 33, further comprising treating said bacterium to facilitate said diffusing into said periplasm.

68. (Withdrawn) The method of claim 67, comprising treating the bacterium with hyperosmotic conditions.

69. (Withdrawn) The method of claim 67, comprising treating the bacterium with physical stress.

70. (Withdrawn) The method of claim 67, comprising treating the bacterium with a phage.

71. (Withdrawn) The method of claim 33, wherein said bacterium is grown at a sub-physiological temperature.

72. (Withdrawn) The method of claim 71, wherein said sub-physiological temperature is about 25°C.

73. (Withdrawn) The method of claim 33, wherein said selecting comprises FACS.

74. (Withdrawn) The method of claim 33, wherein said selecting comprises magnetic separation.

APPENDIX 2: LISTING OF APPEALED CLAIMS FOLLOWING ENTRY OF THE AMENDMENT AND RESPONSE TO FOURTH OFFICE ACTION DATED MAY 4, 2004

1. (Previously presented) A method of obtaining a bacterium comprising a nucleic acid sequence encoding a binding protein capable of binding a target ligand comprising the steps of:

(a) providing a Gram negative bacterium comprising a nucleic acid sequence encoding a candidate binding protein, wherein said binding protein is expressed in soluble form in the periplasm of said bacterium;

(b) contacting said bacterium with a labeled ligand capable of diffusing into said periplasm; and

(c) selecting said bacterium based on the presence of said labeled ligand within the periplasm, wherein said ligand and said candidate binding protein are bound in said bacterium.

2. (Original) The method of claim 1, further defined as a method of obtaining a nucleic acid sequence encoding a binding protein capable of binding a target ligand, the method further comprising the step of:

(d) cloning said nucleic acid sequence encoding said candidate binding protein.

3. (Canceled) The method of claim 1, wherein said binding protein is expressed in soluble form in the periplasm of said bacterium.

4. (Currently amended) The method of claim ~~3~~1, wherein said nucleic acid sequence encoding a candidate binding protein is further defined as operably linked to a leader sequence capable of directing expression of said candidate binding protein in said periplasm.

5. (Original) The method of claim 1, wherein said Gram negative bacterium is an *E. coli* bacterium.

6. (Original) The method of claim 1, further defined as comprising providing a population of Gram negative bacteria.

7. (Original) The method of claim 6, wherein said population of bacteria is further defined as collectively capable of expressing a plurality of candidate binding proteins.

8. (Original) The method of claim 7, wherein said population of bacteria is obtained by a method comprising the steps of:

(a) preparing a plurality DNA inserts which collectively encode a plurality of different potential binding proteins, and

(b) transforming a population of Gram negative bacteria with said DNA inserts.

9. (Original) The method of claim 6, wherein said population of Gram negative bacteria is contacted with said labeled ligand.

10. (Original) The method of claim 1, wherein said candidate binding protein is further defined as an antibody or fragment thereof.

11. (Original) The method of claim 1, wherein said candidate binding protein is further defined as a binding protein other than an antibody.

12. (Original) The method of claim 1, wherein said candidate binding protein is further defined as an enzyme.

13. (Original) The method of claim 1, wherein said candidate binding protein is further defined as not capable of diffusing out of said periplasm in intact bacteria.

14. (Original) The method of claim 1, wherein said labeled ligand comprises a peptide.

15. (Original) The method of claim 1, wherein said labeled ligand comprises a polypeptide.

16. (Original) The method of claim 1, wherein said labeled ligand comprises an enzyme.

17. (Original) The method of claim 1 where said labeled ligand comprises a nucleic acid.

18. (Original) The method of claim 1, wherein said labeled ligand is further defined as comprising a molecular weight of less than about 20,000 Da.

19. (Original) The method of claim 1, wherein said labeled ligand is further defined as comprising a molecular weight of less than about 5,000 Da.

20. (Original) The method of claim 1, wherein said labeled ligand is further defined as comprising a molecular weight of greater than 600 Da and less than about 30,000 Da.

21. (Original) The method of claim 1, wherein said labeled ligand is further defined as fluorescently labeled.

22. (Previously presented) The methods of claim 1, wherein said nucleic acid encoding a candidate binding protein is further defined as capable of being amplified following said selection.

23. (Original) The method of claim 1, further comprising treating said bacterium to facilitate said diffusing into said periplasm.

24. (Original) The method of claim 23, comprising treating the bacterium with hyperosmotic conditions.

25. (Original) The method of claim 23, comprising treating the bacterium with physical stress.

26. (Original) The method of claim 24, comprising treating the bacterium with a phage.

27. (Original) The method of claim 1, wherein said bacterium is grown at a sub-physiological temperature.

28. (Original) The method of claim 27, wherein said sub-physiological temperature is about 25°C

29. (Original) The method of claim 1, further comprising removing labeled ligand not bound to said candidate binding protein.

30. (Previously presented) The method of claim 1, wherein said selecting comprises fluorescent activated cell sorting.

31. (Original) The method of claim 1, wherein said selecting comprises magnetic separation.

32. (Original) The method of claim 1, wherein said ligand and said candidate binding protein are reversibly bound in said periplasm.

33. (Withdrawn) A method of obtaining a bacterium comprising a nucleic acid sequence encoding a catalytic protein catalyzing a chemical reaction involving a target substrate, the method comprising the steps of:

(a) providing a Gram negative bacterium comprising a nucleic acid sequence encoding a candidate catalytic protein, wherein said catalytic protein is expressed in soluble form in said bacterium;

(b) contacting said bacterium with a target substrate capable of diffusing into said bacterium, wherein said candidate catalytic protein catalyzes a chemical reaction involving said target substrate and wherein said chemical reaction yields at least a first substrate product; and

(c) selecting said bacterium based on the presence of said first substrate product.

34. (Withdrawn) The method of claim 33, further defined as a method of obtaining a nucleic acid sequence encoding a catalytic protein catalyzing a reaction with a target substrate, the method further comprising the step of:

(d) cloning said nucleic acid sequence encoding said candidate catalytic protein.

35. (Withdrawn) The method of claim 33, wherein said candidate catalytic protein is expressed in soluble form in the periplasm of said bacterium.

36. (Withdrawn) The method of claim 35, wherein said nucleic acid sequence encoding a candidate catalytic protein is further defined as operably linked to a leader sequence capable of directing expression of said candidate catalytic protein in said periplasm.

37. (Withdrawn) The method of claim 33, wherein said Gram negative bacterium is an *E. coli* bacterium.

38. (Withdrawn) The method of claim 33, further defined as comprising providing a population of Gram negative bacteria.

39. (Withdrawn) The method of claim 38, wherein said population of bacteria is further defined as collectively capable of expressing a plurality of candidate catalytic proteins.

40. (Withdrawn) The method of claim 39, wherein said population of bacteria is obtained by a method comprising the steps of:

(a) preparing a plurality DNA inserts which collectively encode a plurality of different candidate catalytic proteins, and

(b) transforming a population of Gram negative bacteria with said DNA inserts.

41. (Withdrawn) The method of claim 38, wherein said population of Gram negative bacteria is contacted with said target substrate.

42. (Withdrawn) The method of claim 33, wherein said candidate catalytic protein is further defined as an enzyme.

43. (Withdrawn) The method of claim 33, wherein said candidate catalytic protein is further defined as not capable of diffusing out of said periplasm.

44. (Withdrawn) The method of claim 33, wherein said target substrate comprises a molecule containing a scissile amide bond.

45. (Withdrawn) The method of claim 33, wherein said target substrate comprises a polypeptide.

46. (Withdrawn) The method of claim 33, wherein said target substrate comprises a molecule containing a scissile carboxylic ester bond.

47. (Withdrawn) The method of claim 33, wherein said target substrate comprises a nucleic acid.

48. (Withdrawn) The method of claim 33, wherein said target substrate comprises a molecule containing a scissile phosphate ester bond.

49. (Withdrawn) The method of claim 33, wherein said target substrate comprises a molecule containing a scissile sulfonate ester bond.

50. (Withdrawn) The method of claim 33, wherein said target substrate comprises a molecule containing a scissile carbonate ester bond.

51. (Withdrawn) The method of claim 33, wherein said target substrate comprises a molecule containing a scissile carbamate bond.

52. (Withdrawn) The method of claim 33, wherein said target substrate comprises a molecule containing a scissile thioester bond.

53. (Withdrawn) The method of claim 33, wherein said target substrate is further defined as comprising a molecular weight of less than about 20,000 Da.

54. (Withdrawn) The method of claim 33, wherein said target substrate is further defined as comprising a molecular weight of less than about 5,000 Da.

55. (Withdrawn) The method of claim 33, wherein said target substrate is further defined as comprising a molecular weight of less than about 3,000 Da.

56. (Withdrawn) The method of claim 33, wherein said target substrate is further defined as comprising a molecular weight of greater than about 600 Da and less than about 30,000 Da.

57. (Withdrawn) The method of claim 33, wherein said first substrate product is further defined as capable of being detected based on the presence of a fluorescent signature.

58. The method of claim 57, wherein said fluorescent signature is absent in said target substrate.

59. (Withdrawn) The method of claim 58, wherein said fluorescent signature is produced by catalytic cleavage of a scissile bond.

60. (Withdrawn) The method of claim 59, further defined as comprising use of a FRET system, said FRET system comprising a fluorophore bound by a scissile bond to at least a first molecule capable of quenching the fluorescence of said fluorophore, wherein cleavage of said scissile bond allows said first molecule to diffuse away from the fluorophore and wherein the fluorescence of said fluorophore becomes detectable.

61. (Withdrawn) The method of claim 60, wherein the fluorophore comprises a positive charge allowing the fluorophore to remain associated with the bacterium.

62. The method of claim 57, wherein said target substrate is further defined as comprising a latent fluorescent moiety capable of being released by said chemical reaction involving said target substrate.

63. (Withdrawn) The method of claim 62, wherein the latent fluorescent moiety released by said cleavage possesses an overall positive charge allowing said moiety to remain associated with the bacterium following said cleavage.

64. (Withdrawn) The method of claim 57, further defined as comprising labeling said target substrate with a fluorescent pH probe capable of being detected upon a change in pH associated with said chemical reaction involving said target substrate.

65. (Withdrawn) The method of claim 64, wherein said fluorescent pH probe possesses an overall positive charge allowing said fluorescent pH probe to remain associated with the bacterium following said chemical reaction involving said target substrate.

66. (Withdrawn) The method of claim 33, wherein said bacterium is further defined as viable following said selecting.

67. (Withdrawn) The method of claim 33, further comprising treating said bacterium to facilitate said diffusing into said periplasm.

68. (Withdrawn) The method of claim 67, comprising treating the bacterium with hyperosmotic conditions.

69. (Withdrawn) The method of claim 67, comprising treating the bacterium with physical stress.

70. (Withdrawn) The method of claim 67, comprising treating the bacterium with a phage.

71. (Withdrawn) The method of claim 33, wherein said bacterium is grown at a sub-physiological temperature.

72. (Withdrawn) The method of claim 71, wherein said sub-physiological temperature is about 25°C.

73. (Withdrawn) The method of claim 33, wherein said selecting comprises FACS.

74. (Withdrawn) The method of claim 33, wherein said selecting comprises magnetic separation.

APPENDIX 3: EVIDENCE APPENDIX

Exhibit A: Declaration of Jongsik Gam; cited in Applicants' Amendment and Response to Office Action Dated March 4, 2004, filed May 4, 2004

Exhibit B: Chen *et al.*, *Nature Biotechnology,* 19:537-542 (2001); submitted as IDS reference C54

Exhibit C: Merriam-Webster™ Online Dictionary definition of "diffuse"; cited in Applicants' Amendment and Response to Office Action Dated March 4, 2004, filed May 4, 2004

Exhibit D: Cambridge Online Dictionary™ (http://dictionary.cambridge.org/) definition of "diffuse"; cited in Applicants' Amendment and Response to Office Action Dated March 4, 2004, filed May 4, 2004

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
Gang Chen, et al.

Serial No.: 09/699,023

Filed: October 27, 2000

For: ISOLATION OF BINDING PROTEINS WITH HIGH AFFINITY TO LIGANDS

Group Art Unit: 1632

Examiner: Ford, V.

Atty. Dkt. No.: UTSB:675US

CERTIFICATE OF MAILING
37 C.F.R. §1.8

I hereby certify that this correspondence is being deposited with the U.S. Postal Service with sufficient postage as First Class Mail in an envelope addressed to: Assistant Commissioner for Patents, Washington, DC 20231, on the date below:

05/04/04
Date

Robert E. Hanson

DECLARATION OF JONGSIK GAM UNDER 37 C.F.R. § 1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

I, JONGSIK GAM, HEREBY DECLARE AS FOLLOWS:

1. I am a citizen of South Korea, and currently reside at 1014 Stratford Ct., State College, PA.

2. I was employed by The University of Texas from 1997 until 2004, with the title of Research Assistant. During that time I was a graduate student at The University of Texas. My primary duties as a Research Assistant were in conducting scientific research in the area of

pharmacy. One of my two supervisors was Brent Iverson, Ph.D., who is a co-inventor of the above captioned patent application.

2. I will receive a Ph.D. in Pharmacy from The University of Texas in May, 2004. I have been conducting research in the area of pharmacy since 1997. I have a Bachelors degree and a Masters degree in Pharmacy from the Seoul National University in Seoul, South Korea.

3. I have reviewed the amended claims of the above-captioned patent application and am familiar with the technology and steps described in the claims.

4. I understand that the Patent and Trademark Office Examiner in charge of assessing the patentability of the referenced patent application has rejected the claims of the above patent application for failing to recite a wash step. In particular, it is my understanding that the Examiner asserts that a separate wash step must be added to make the following procedure functional:

> 1. (Current amended) A method of obtaining a bacterium comprising a nucleic acid sequence encoding a binding protein capable of binding a target ligand comprising the steps of:
> (a) providing a Gram negative bacterium comprising a nucleic acid sequence encoding a candidate binding protein, wherein said binding protein is expressed in soluble form in the periplasm of said bacterium;
> (b) contacting said bacterium with a labeled ligand capable of entering said periplasm; and
> (c) selecting said bacterium based on the presence of said labeled ligand within the periplasm, wherein said ligand and said candidate binding protein are bound in said bacterium.

5. I am providing the present Declaration to submit data demonstrating that a wash step is not required for the successful use of the above procedure.

6. The studies carried out by me demonstrating that a wash step is not necessary for the function of the technique described in the claims can be summarized as follows:

A. Bacteria

E. coli ABLE™C and TG1 bacteria were obtained expressing a digoxigenin-specific scFv in soluble form in the periplasm. Periplasmic expression was achieved by linking the scFv to a *pelB* leader sequence. Expression was regulated by the *lac* inducible promoter, the expression of which is induced by the addition of IPTG to growth media.

B. Cell culture

Growth media was supplemented with ampicillin (100 μg/ml) when necessary. Bacteria containing the plasmid encoding the scFv were grown overnight in TB broth containing 2% glucose at 30°C. Cells were subcultured in fresh 2xYT (or LB) media and re-grown at 37°C for 2 hours until an OD600 of 0.5-0.8 was reached. The cells were induced with IPTG (0.2 mM) for an additional 4 hours at 25°C to express the scFv antibodies in the periplasm of the *E. coli* strains.

C. Labeling

50 μl of the induced cell cultures were incubated in 950 μl of 1xPBS at room temperature for 1 hour with BODIPY-conjugated digoxigenin (usually 200 nM = 0.2 μM). The incubation was performed successfully with and without use of permeabilizers (PMBN, EDTA or lactic acids were used when a permeabilizer was included). At this stage, trials were run both with and without a washing step after the incubation. The resulting cell suspension (10 μl without a washing step), was diluted with 1 ml of 1xPBS in a SIP tube for the FACS analysis.

D. Selection

10^4 events were analyzed on a Becton Dickinson FACSCalibur for both the washed and unwashed trials. Sheath flow was 1xPBS for all of the screens. Cell viability was judged using the propidium

iodide (PI) staining method (1 μg/ml). The screening demonstrated a high FACS signal relative to negative controls lacking the digoxigenin-specific scFv both with and without the wash step. The results confirmed that in cells expressing the scFv binding protein with specific affinity for the labeled ligand the bound complex was accumulated and becomes detectable above background levels (the control) based on this concentration of signal regardless of the presence of unbound labeled ligand.

7. Based on the foregoing studies, a wash step is not required for the use of the method described in the claims of this patent application to obtain a bacterium comprising a nucleic acid sequence encoding a binding protein capable of binding a target ligand.

8. I hereby declare that all statements made herein of my knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

5/4/04

Date

Jongsik Gam

Isolation of high-affinity ligand-binding proteins by periplasmic expression with cytometric screening (PECS)

Gang Chen[1†], Andrew Hayhurst[1,2†], Jeffery G. Thomas[2,3], Barrett R. Harvey[1], Brent L. Iverson[1,4*], and George Georgiou[1,2,5*]

Periplasmic expression with cytometric screening (PECS) is a powerful and rapid "display-less" technology for isolating ligand-binding proteins from diverse libraries. *Escherichia coli* expressing a library of proteins secreted into the periplasmic space are incubated with a fluorescent conjugate of the target ligand. Under the proper conditions, ligands as large as about 10 kDa can equilibrate within the periplasmic space without compromising the cell's integrity or viability. The bacterial cell envelope effectively serves as a dialysis bag to selectively retain receptor–fluorescent probe complexes but not free ligand. Cells displaying increased fluorescence are then isolated by flow cytometry. We demonstrate that scFv antibodies with both very high and low affinity to digoxigenin can be isolated from libraries screened by PECS using a benchtop flow cytometer. We also show that preexisting libraries constructed for display on filamentous bacteriophage can be screened by PECS without the need for subcloning. In fact, PECS was found to select for proteins that could be missed by conventional phage panning and screening methods.

Protein display technologies collectively represent one of the most powerful tools for protein engineering[1]. For display purposes, a protein is fused to the C or N terminus of a polypeptide sequence that targets the resulting chimera onto the surfaces of biological particles such as viruses, bacteria, yeast, or higher cells. Libraries are typically screened for ligand binding by a series of adsorption–desorption cycles, a process also known as "panning"[2]. Panning has been used successfully to screen highly complex libraries made by cloning the mammalian antibody repertoire and displaying it on phage (up to 10^{11} clones). For somewhat less diverse libraries (up to 10^9 clones), display on bacteria or yeast coupled with flow cytometry is a powerful tool for the discovery of proteins with exceptionally high ligand-binding affinities and for the isolation of enzyme catalysts[3–5].

Although the importance of display technologies for protein engineering is undisputed, the need to anchor the target polypeptide onto the surface of a biological particle imposes a number of limitations that can significantly reduce the diversity of the library relative to the totality of proteins that can be produced in a soluble form within the cell. First and foremost, protein display requires that the protein of interest be expressed as either a C- or N-terminal fusion, a process that can adversely affect protein function or stability. Second, protein display is subject to biological constraints associated with protein export and presentation, which may compromise the viability of the virus or cell (e.g., refs 6,7). Third, complex proteins consisting of several polypeptide chains or requiring certain post-translational modifications may be intrinsically incompatible with display on phage, bacteria, or yeast. Such proteins include, for example, cofactor-containing bacterial proteins whose biosynthesis depends on the recently discovered twin arginine motif export system[8]. Fourth, display can introduce certain complications for screening such as avidity effects[9–11], in the case of viruses, or nonspecific binding of the fluorescent probe to the cell surface[6].

Here we describe a "display-less" approach for isolating proteins that bind to small molecules, including peptides and oligonucleotides, based on PECS. Libraries are simply expressed in soluble form in the periplasmic space of *E. coli* by means of a suitable signal sequence and mixed with a fluorescently tagged ligand (Fig. 1). Normally, the outer membrane of Gram-negative bacteria restricts the diffusion of molecules larger than ~650 Da[12]. However, we have found that an appropriate combination of growth conditions and bacterial strains permits molecules as large as 10 kDa to equilibrate through the outer membrane while periplasmically expressed proteins are retained within the cell. Specific binding of the fluorescent conjugate by the cognate periplasmic protein results in increased fluorescence, allowing the cells to be isolated from the rest of the library by fluorescence-activated cell sorting (FACS). Thus the cell envelope serves as a dialysis bag that retains protein–ligand complexes.

We have demonstrated the utility of PECS for discovering ligand-binding scFv antibodies. One advantage of PECS is that it can readily be used to screen by flow cytometry any existing library constructed for display on bacteriophage without the need for subcloning. This feature allowed the isolation of unique binding clones that could not be identified by conventional phage panning.

Results and discussion

Fluorescence detection and enrichment of cells expressing scFv antibodies in the periplasm. The 26-10 scFv antibody binds with high affinity to cardiac glycosides such as digoxin and digoxigenin (the K_d values of the purified antibody for digoxin and digoxigenin are $0.9 \pm 0.2 \times 10^{-9}$ M and $2.4 \pm 0.4 \times 10^{-9}$ M, respectively[13]). The 26-10 scFv and its variants have been used extensively as a model system to understand the effect of mutations in the complementarity-determining regions (CDRs) and in the framework regions on hapten binding[4,13–16]. We expressed the 26-10 scFv in the *E. coli* periplasm

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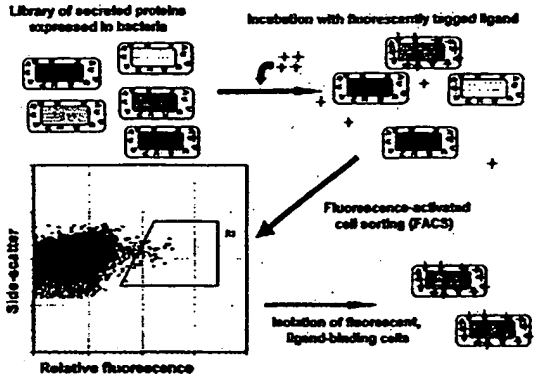

Figure 1. A schematic diagram showing the principle of periplasmic expression with cytometric screening (PECS). Libraries of proteins secreted to the *E. coli* periplasm can be labeled by fluorescent conjugates of ligands by a simple high-salt incubation procedure. The outer membrane serves as a dialysis membrane allowing diffusion of unbound ligand out of the cell and retention of protein–ligand complexes within the cell. The fluorescent cells are then separated by FACS according to user-defined parameters (e.g., region one: R1). Only cells expressing protein species with the desired ligand-binding characteristics are enriched.

using the arabinose promoter and *pelB* leader sequence in plasmid pBAD30pelB-Dig. This vector was used to transform the *ara*⁻ *E. coli* strain LMG194, and scFv expression was induced with 0.2% (wt/vol) arabinose at 25°C. We observed that upon incubation with 100 nM of digoxigenin-BODIPY, the cells became strongly fluorescent, and the fluorescence signal was retained even after extensive washing to remove nonspecifically bound ligand (data not shown). Staining with the viability stain propidium iodide (PI), which binds specifically to damaged cells by intercalating in normally inaccessible double-stranded nucleic acids, revealed that >90% of the cells were not permeable to the dye. The fraction of viable cells was similar to that in control cultures harvested in late exponential phase, indicating that the ligand-bound *E. coli* cells were structurally intact.

Cells expressing the 26-10 scFv antibody in the periplasm could be enriched from a large excess of *E. coli* transformed with vector alone in a single round of sorting. Specifically, LMG194 (pBAD30pelB-Dig) cells were mixed with a 10,000-fold excess of *E. coli* containing vector devoid of an scFv insert (pBAD30). After fluorescence labeling and cell sorting, enrichment factors of over 1,000 fold per round were obtained (data not shown). Cells expressing control scFv as opposed to empty vector were also unstained by the labeling procedure (see below and Fig. 2).

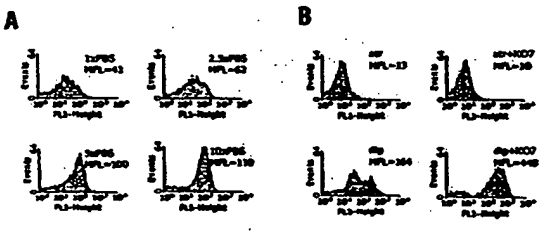

Figure 2. Optimization of the periplasmic expression screening signal. (A) Effect of hyperosmotic shock on efficiency of labeling of the 26-10 scFv expressed from pHEN2.dig26-10 in *E. coli* ABLEC and labeled for 1 h with 100 nM digoxigenin-BODIPY. The PBS concentration employed and the mean fluorescence (MFL) of the populations are shown. Cells expressing unrelated antibodies (to atrazine or thyroglobulin) and labeled under identical conditions gave a MFL of 5–6 (data not shown). (B) Effect of infection with M13K07 on the fluorescence signal detected in ABLEC cells labeled with 100 nM digoxigenin-BODIPY in 5× PBS. Cells were expressing either an anti-atrazine scFv (atr) or the 26-10 scFv (dig) using the powerful P_{tac} promoter and either left uninfected or infected (K07) at a multiplicity of infection of 10 at 0.5 h preinduction with 1 mM IPTG and selection for phage infection with kanamycin (70 μg/ml).

Table 1. Light-chain CDR3 amino acid sequences of mutants[a]

	Light-chain sequence	Off-rate (s^{-1})
	90 96	
A4-19	Q T T H V P P	2.7×10^{-4}
60.1 (1)	Q T T H S P A	5.5×10^{-4}
60.2 (2)	Q T T H L P T	2.8×10^{-4}
60.3 (1)	Q T T H T P P	ND
60.4 (1)	Q T T H L P A	ND
90.1 (1)	Q T T H I P T	3.2×10^{-4}
90.2 (1)	Q T T H V P P	2.7×10^{-4}
90.3 (7)	Q T T H V P A	2.2×10^{-4}
90.4 (1)	Q T T H I P A	1.4×10^{-4}
90.5 (3)	Q T T H L P A	ND
90.6 (1)	Q T T H V P C	ND

[a]Mutants were isolated by 60 min (60.1–60.4) and 90 min (90.1–90.6) off-rate selection with the actual number of identical clones isolated in parentheses. ND, No data.

Antibody affinity maturation using PECS. An affinity-improved variant of the 26-10 scFv containing three amino acid substitutions in heavy-chain CDR1 (T30P, D31S, and M34Y) was isolated[15]. This clone was designated A4-19. Using surface plasmon resonance, we determined the equilibrium dissociation constant (K_D) of the purified, monomeric A4-19 scFv to be 0.3 nM compared to 0.9 nM for the 26-10 scFv parental antibody. Three light-chain CDR3 residues that make contact with (Thr91, Pro96) or are in close proximity to (Val94) the digoxin hapten[17] were randomized using an NNS (S = G or C) strategy[16]. A library of 10^6 transformants was obtained in the pBADpelB vector. The library was screened by two rounds of FACS using a low-throughput benchtop flow cytometer (Becton Dickinson FACSort). In the first round of screening, the labeled cells were washed once with PBS and sorted in recovery mode, which collects all fluorescent events including droplets that contain both a nonfluorescent and fluorescent particle. Operation in recovery mode collects more rare cells at the expense of purity. The collected cells were regrown, labeled, washed, and then incubated with a 50-fold excess (5 μM) of free digoxin for various times. Cells that retained the desired level of fluorescence were isolated by FACS using exclusion mode, which ensures a higher degree of purity by rejecting coincident fluorescent and nonfluorescent events. Five random clones from the cell population isolated following incubation with competitor for 60 min and 13 clones from the 90 min pool were picked at random and sequenced (Table 1). The respective scFv antibody proteins were purified by Ni affinity chromatography followed by gel filtration fast-performance liquid chromatography (FPLC), and the hapten-binding kinetics of the monomeric scFv proteins were determined by surface plasmon resonance. All the scFvs examined displayed association rate constants (k_{on}) indistinguishable from those of the starting A4-19 antibody ($0.9 \pm 0.2 \times 10^6\ M^{-1}s^{-1}$). The k_{off} of the clones isolated after 60 min of competition were the same as or faster than that of A4-19. However, two out of three clones isolated after a 90 min competition exhibited slower k_{off} values, with one clone, 90.4, having a twofold slower dissociation rate constant resulting in a K_D of 150 pM. Thus, we could isolate a higher affinity mutant of an antibody that already exhibited a subnanomolar K_D by screening a library of 10^6 clones using only two rounds of PECS.

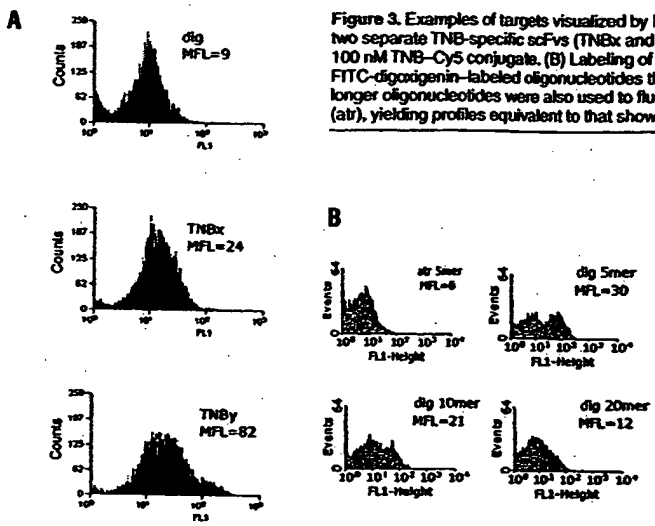

Figure 3. Examples of targets visualized by PECS. (A) Fluorescence distribution of ABLEC cells expressing two separate TNB-specific scFvs (TNBx and TNBy) and the control 26-10 scFv (dig) labeled in 5× PBS with 100 nM TNB–Cy5 conjugate. (B) Labeling of cells expressing the 26-10 scFv (dig) with 100 nM FITC-digoxigenin–labeled oligonucleotides that are 5 (5mer), 10 (10mer), or 20 (20mer) bases long. The longer oligonucleotides were also used to fluorescence-label cells expressing the anti-atrazine control scFv (atr), yielding profiles equivalent to that shown for the 5mer.

Optimization of labeling conditions and application to other ligands. To achieve optimal enrichment ratios during library screening by PECS, it is important to obtain the maximum fluorescence signal possible without compromising cell viability[4,18]. The fluorescent intensity of cells expressing binding proteins in the periplasm and incubated with ligand concentrations above the K_D was found to depend on a number of physiological and genetic parameters. A significant increase in fluorescence with concomitant retention of cell viability was observed when the cells were grown at 25°C. Little soluble scFv is produced at 37°C (e.g. ref. 19), and growth at subphysiological temperatures generally enhances the yield of correctly folded proteins. Importantly, growth at 25°C increases the permeability of the outer membrane, in part because the cold-sensitive secretory pathway alters the membrane composition[20].

Various *E. coli* strains expressing the 26-10 scFv exhibited significant differences with respect to labeling with 100 nM digoxigenin-BODIPY. For example, the commonly used library strains TG-1 and HB2151 displayed a mean fluorescence intensity only threefold higher relative to the respective control cells containing vector alone. The highest signal (eightfold higher than the control) was obtained with *E. coli* ABLEC under a variety of induction regimes and growth conditions. In all media (LB, TB, 2× TY), at all temperatures (20°, 25°, and 30°C), with all combinations of temperature shifts upon induction (37°–25°, 37°–30°C, no shift), and with all levels of IPTG induction (10, 100, 1,000 μM), ABLEC surpassed the other strains tested.

The ionic strength and composition of the buffer used for labeling also had a pronounced effect on the mean fluorescence intensity of the cells (Fig. 2A). Labeling in 5-fold or 10-fold concentrated PBS resulted in a more uniform and highly fluorescent population. Labeling in 5× PBS gave a peak-to-peak separation between control and 26-10 scFv-expressing cells of around 20-fold. The elevated salt concentration and osmotic pressure under these conditions may be disrupting the ordered nature of the lipopolysaccharide layer in the outer membrane, resulting in increased permeability[21,22]. Whereas 10× PBS gave the highest mean fluorescence intensity, cell viability (as judged by PI staining) tended to be compromised, and so we typically used 5× PBS. The highest fluorescent signals were obtained by (1) driving the expression of scFv antibodies from the more powerful P_{tac} promoter; (2) using ABLEC as the host strain; (3) labeling in 5× PBS buffer; and (4) infecting the cells with the filamentous phage M13K07 (Fig. 2B). Although pIII (a minor coat protein) expressed in the absence of other phage proteins has been shown to increase the permeability of the outer membrane[23], overexpression of pIII is extremely toxic to the host cell, especially when it is fused to scFv (ref. 24). Likewise, although mutants of the large aqueous pore-forming pIV have been shown to improve permeability to ligands above the 650 Da limit[25], its overexpression induces the phage shock response[26], adversely affecting cell growth. Thus, although phage infection resulted in the highest fluorescent intensities without reducing cell viability, we generally avoided it because of much slower growth of infected versus uninfected cells.

Using optimal conditions, cells expressing scFvs in the periplasm could be fluorescently stained with a variety of ligands including small molecules, oligonucleotides, or peptides with molecular weights as high as 10,000 Da (Fig. 3). For example, Figure 3A shows the fluorescence distribution of cells expressing two different scFv antibodies to trinitrobenzene (TNB) isolated from a naïve library (A. Hayhurst, B.R. Harvey, B.L. Iverson, and G. Georgiou, unpublished results). The cells were stained with the fluorescent conjugate

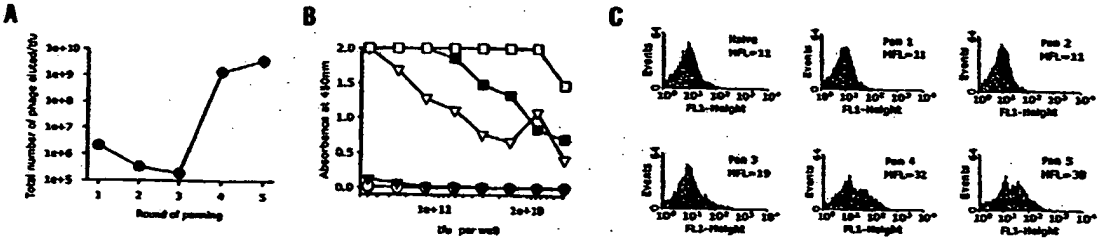

Figure 4. Monitoring the phage panning process using FACS via PECS. (A) Phage eluate titers after each round of panning the Griffin library on digoxin–BSA conjugate. tfu, Transforming units. (B) Polyclonal phage ELISA of purified phage stocks from each round of panning on digoxin–ovalbumin. Points are the average of duplicate wells. The naïve signal is overshadowed by the pan1 signal. Naïve library (filled circles); pan 1(empty circles); pan 2 (inverted filled triangles); pan 3 (inverted empty triangles); pan 4 (filled squares); pan 5 (empty squares). (C) Periplasmic expression screening of a portion of the naïve Griffin library and rounds 1 to 5 from a panning experiment following infection of ABLEC and labeling in 5× PBS with 100 nM digoxigenin-BODIPY.

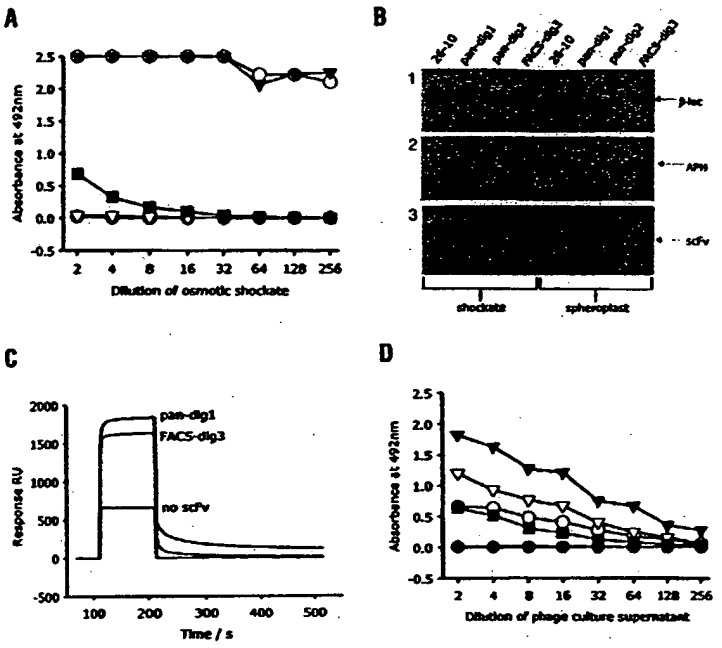

Figure 5. Examining clones isolated by phage display and PECS. (A) Soluble scFv ELISA signals from osmotic shock fluid of ABLEC containing pHEN2 with negative control anti-atrazine scFv (filled circles); positive control 26-10 (empty circles); pan-dig1 (inverted filled triangles); pan-dig2 (inverted empty triangles); FACS-dig3 (filled squares). One-tenth of the amount of shockate isolated from 20 ml OD units/cm at A_{600} (or 100 µl) was twofold serially diluted in duplicate on digoxigenin-ovalbumin–coated ELISA wells. (No binding to ovalbumin alone was detected using these conditions.) (B) Western blot of osmotic shockate and spheroplast fractions of equal amounts of cells (the same samples used for derivation of shockates used in Fig. 4A) probed with (1) anti-β-lactamase (β-lac) serum (2) anti-aminoglycoside phosphotransferase (APH) serum, and (3) anti-his^6 serum (scFv). (1) and (2) were used as controls to demonstrate the efficiency of fractionation. Anti-rabbit-HRP (1 and 2) and anti-mouse-HRP (3) were used as secondary antibodies. (C) BIAcore analysis of 500 µM purified monomeric pan-dig1 and FACS-dig3 scFv on a digoxin-BSA–coupled chip. Sensorgrams were subtracted from a BSA blank. (D) Monoclonal phage ELISA on digoxin-ovalbumin of culture supernatants from M13K07 infected TG-1–bearing negative control pHEN2.atr (filled circles); pHEN2.26-10 (empty circles); pHEN2.pan-dig1 (filled inverted triangles); pHEN2.pan-dig2 (empty inverted triangles); pHEN2.FACS-dig3 (filled squares). Infected cells were grown with vigorous aeration at 25°C overnight in glucose-free 2× TY, cells pelleted, and 100 µl of supernatant twofold serially diluted. (No binding to ovalbumin alone under these conditions was detected.)

cyanine 5–diaminopentane–trinitrobenzene (Cy5-DAP-TNB), which is positively charged and has a molecular weight of 953 Da. The best clone (TNBy) exhibited a ninefold higher mean fluorescence relative to cells expressing the 26-10 scFv antibody that does not recognize the TNB hapten. Similarly, cells expressing a scFv specific for a 21-mer peptide could be labeled with the respective fluorescent peptide conjugate and affinity-matured using PECS (G. Chen, A. Hayhurst, B.R. Harvey, B.L. Iverson, and G. Georgiou, unpublished results).

To evaluate the molecular weight limit for the diffusion of ligands into the periplasmic space under the conditions identified above, we used a set of oligonucleotides bearing digoxigenin at one end and a fluorescein isothiocyanate (FITC) label at the other end. The oligonucleotides were synthesized using phosphorothioate linkages to minimize degradation by nucleases and consisted of 5 (2,384 Da), 10 (4,897 Da), or 20 (8,727 Da) adenosine residues. Upon incubation with 100 nM probe, negative control cells expressing a scFv to the unrelated hapten atrazine[27] showed background fluorescence distribution (mean fluorescence intensity ~6) irrespective of the size of the probe. In contrast, cells expressing the 26-10 scFv, which binds to the digoxigenin moiety of the oligonucleotides, exhibited 5-fold, 3.5-fold, and ~2-fold greater fluorescence than the control when labeled with the 5-mer, 10-mer, or 20-mer, respectively (Fig. 3B). Presumably, as the size and charge of the oligonucleotides increase, we are reaching the limits on outer membrane permeability under the conditions we used. It should be noted, however, that in our hands a twofold fluorescence difference still allows resolution and sorting of populations.

Analysis and screening of phage libraries by FACS. We have found that FACS can be used to isolate ligand-binding proteins from existing libraries constructed for display on bacteriophage. This is very useful, because the construction of highly diverse libraries *de novo* is technically demanding. Phage-displayed libraries are amenable to screening by PECS because, during phage biogenesis, pIII fusions are first targeted to the periplasm and anchored onto the inner membrane by a small C-terminal portion of pIII (refs 28,29). In the most widely used vectors for phage display, an amber codon is placed between the N-terminal scFv and the pIII gene. Thus, as a consequence of inefficient amber suppression, all libraries containing amber codons give some periplasmic expression of the proteins not fused to pIII.

Conventional phage panning of a widely used semisynthetic library (Griffin. 1) successfully enriched digoxin-binding phage (Fig. 4A, B). Pans 3 and 4 had 0/48 and 28/48 positives, respectively, as judged by monoclonal phage enzyme-linked immunosorbent assay (ELISA). Twenty-four positive clones from pan 4 shared the same DNA fingerprint. DNA sequencing of six isolates confirmed the presence of a single heavy- and light-chain family used (VH5 and Vλ1). One clone was represented five times ("pan-dig1"), whereas a second was found once ("pan-dig2"). Mutations responsible for differences between pan-dig1 and pan-dig2 were in HCDR3 (P97T, S98Y), LCDR3 (R95aP, A95bR, V96-), and LFR1 (L4V).

The phage population from each round was used to infect ABLEC. Upon labeling with digoxigenin-BODIPY, the cell populations from each round displayed fluorescence distributions consistent with the corresponding phage ELISA signals (compare Fig. 4A and C). Significant enrichment of binding clones in a single round of PECS was obtained with cell populations infected with phage from the third and fourth rounds of panning. The fourth round yielded 7/8 positive for hapten binding by FACS, all clones being identical to pan-dig1. Although the third round yielded 5/14 FACS positives, 3/5 encoded a novel scFv, designated "FACS-dig3" (the remaining 2/5 were pan-dig1). This clone exhibited a completely different DNA fingerprint compared to the clones isolated by panning. Sequencing revealed a different heavy chain (VH3) and minor variations in LCDR3 (R95aG, A95bG, V96P) relative to pan-dig1. Importantly, the FACS-dig3 DNA fingerprint was never detected in two separate phage panning experiments involving the analysis of over 350 phage clones from rounds 3,

4, and 5 (those that exhibited a positive signal by conventional monoclonal phage ELISA being fingerprinted). Thus, FACS-dig3 represents a library clone that could apparently only be identified by PECS, presumably for the reasons detailed below.

The pan-dig1, pan-dig2, and FACS-dig3 scFvs were expressed in ABLEC under identical conditions. Cells expressing pan-dig2 gave no FACS signal, whereas pan-dig1 and FACS-dig3 gave a comparable fluorescence signal (mean fluorescence intensities of 34 and 42, respectively). In ELISA assays of periplasmic (osmotic shock) fractions, pan-dig1 gave a signal comparable to the 26-10 scFv antibody and FACS-dig3 gave a weak signal, whereas pan-dig 2 gave no signal (Fig. 5A). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and western blotting of the same shockate samples used in the ELISA revealed that the absence of an ELISA signal by the pan-dig2 clone was due to the exclusive accumulation by the respective scFv protein in an insoluble form in the cell. The pan-dig1 scFv accumulated at moderate levels in the periplasmic fraction, whereas the clone isolated only by PECS (FACS-dig3) was expressed extremely well and was fully soluble (Fig. 5B). BIAcore analysis of purified, monomeric dig1 and dig3 scFv protein (Fig. 5C) indicated that the latter exhibits a faster hapten dissociation rate. This finding is consistent with the lower signal observed by phage ELISA with the FACS-dig3 clone (Fig. 5A). These results suggest that (1) PECS can only select clones that exhibit at least a threshold of soluble protein expression in bacteria. Such clones are, of course, most desirable for protein engineering applications. (2) Both low- and high-affinity binders can be isolated by a single PECS setup. In contrast, with phage display, higher affinity binders are isolated by monovalent (gene III) display whereas lower affinity binders generally require polyvalent (gene VIII) display. By not relying on display and adsorption for selection, PECS most likely avoids "avidity effects" that often hamper concise phage selections.

To analyze why the FACS-dig3 clone was missed in the panning experiments, pHEN2 bearing the corresponding gene was moved into TG-1 cells. TG-1 cells transformed with phagemids encoding pan-dig1, pan-dig2, FACS-dig3, 26-10 as a positive control, and the anti-atrazine scFv as a negative control, were infected with helper phage and incubated overnight at 25°C. Rescued phage were then analyzed by ELISA (Fig. 5D). FACS-dig3 gave a lower monoclonal phage ELISA signal, indicating that, when expressed as a fusion to pIII, either phage growth or hapten binding is impaired. Interestingly, a similarly lower ELISA signal was also obtained with the 26-10 scFv, even though the latter is well expressed and possesses a high hapten affinity. Thus, whereas FACS-dig3, and for that matter clones expressing the 26-10 scFv, bind to hapten and are enriched, they are ultimately missed because they give a low phage ELISA signal.

Conclusions. PECS is based on the observation that, under certain experimental conditions, fluorescently tagged ligands including synthetic small molecules, peptides, and oligonucleotides can permeate the bacterial outer membrane and become selectively concentrated when bound by a receptor protein within the periplasm. Fluorescent cells can then be isolated by flow cytometry[1,30].

The finding that large ligands can be made to diffuse through the bacterial outer membrane without significant loss of proteins from the periplasm is surprising, but not unprecedented. In fact, there exist physiological mechanisms whereby proteins (e.g., colicins) and even very large complex structures (e.g., filamentous phage) can cross the outer membrane in a highly selective manner. Thus, with further optimization, PECS might be used to screen for binders to larger ligands than those studied here. The system could also be applied to nucleic acid–binding proteins, potentially overcoming the toxicity of these proteins when expressed in the cytoplasm of *E. coli* by other directed evolution methods.

In this report, we have shown that PECS can be used to isolate binders with subnanomolar dissociation constants. Importantly, existing phage display libraries can be screened by PECS. Clones that would have been missed by standard panning and screening approaches, particularly very well-expressed clones, are readily isolated in this manner. By definition, PECS selects only for clones that exhibit good expression characteristics in the host. We are currently using PECS for the isolation of scFvs to small molecules other than digoxin, for the affinity maturation of anti-peptide antibodies, and, finally, for expression maturation of toxic or aggregation-prone proteins (A. Hayhurst, B.R. Harvey, B.L. Iverson, and G. Georgiou, unpublished results). Finally, it should be noted that the work described here was performed using a low-throughput benchtop flow cytometer. With a high-throughput flow cytometer (e.g., a Cytomation MoFlo) or perhaps by employing highly parallel microfluidic sorters[31], it will be possible to screen very large libraries including mammalian complementary DNA libraries and naïve antibody repertoires. The screening of such libraries can be performed using repeated rounds of PECS without intermediate amplifications of the sorted cells, thus affording significant timesaving over phage panning.

Experimental protocol

Fluorescent probes and conjugates. The synthesis of digoxigenin-BODIPY has been described[13,32]. The fluorescent conjugate of TNBS was a derivative of Cy5 (Amersham Pharmacia, Piscataway, NJ). Conjugates of TNB and protein were made by combining 100-fold molar excess of TNBS with ovalbumin or BSA, allowing the mixture to react at room temperature for 2 h, and then dialyzing extensively against PBS. Phosphorothioate linkage oligonucleotides bearing a 5′-digoxigenin ester and a 3′-FITC were from Integrated DNA Technologies (Coralville, IA) and supplied at reverse-phase high-performance liquid chromatography (RP-HPLC) purity.

Strains and plasmids. *Escherichia coli* strains TG1 and HB2151 were provided by the Griffin library. *Escherichia coli* ABLEC was purchased from Stratagene (La Jolla, CA), and helper phage M13K07 was purchased from Amersham Pharmacia. *Escherichia coli* LMG194 (*ara*-) and plasmid pBAD30 were gifts from Jon Beckwith. Plasmid pET25b was from Novagen (Madison, WI). Information on the construction of expression vectors and restriction maps of the plasmids used in this work can be provided by the authors upon request.

Affinity maturation of the A4-19 scFv. A library of 10^6 transformants in which LCDR3 residues Thr91, Val94, and Pro96 in the A4-19 anti-digoxin scFv had been randomized using synthetic oligonucleotides[16] was created. The cells were grown at 37°C overnight and were then subcultured in fresh Luria–Bertani (LB) medium and grown at 25°C for 2 h. The cells were induced with 0.2% arabinose for 4 h and then were incubated with digoxigenin-BODIPY (100 nM) for 1 h. After washing once in PBS buffer, labeled cells were sorted on a Becton Dickinson FACSort (San Jose, CA). The cells were first sorted in recovery mode, collected in PBS and subsequently poured into SOC medium for overnight growth at 37°C. The diluted cells were grown at 25°C and induced with arabinose for 4 h. The cells were labeled with 100 nM probe, then incubated with 5 μM of unlabeled digoxin for between 15 and 90 min and sorted in exclusion mode. Again, the cells were grown in SOC overnight at 37°C and individual clones subsequently selected from plating dilutions of the saturated culture.

Surface plasmon resonance. The association constants of scFv mutants were measured according to the method of Chen *et al.*[17] using a BIAcore 1000 instrument.

PECS Optimization. An aliquot of ABLEC bearing the clone of interest was scraped from a glycerol stock into 1 ml of 2× TY (2% glucose, 100 μg/ml ampicillin) to obtain an OD_{600} of ~0.1. After 2 h of growth at 37°C with vigorous shaking, scFv expression was induced with 1 mM isopropyl-β-D-galactoside (IPTG), and the culture was transferred to 25°C for 4 h. Fifty microliters of culture were labeled with 100 nM of fluorescent probe in 1-ml of 1× PBS (10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 2.7 mM KCl, 137 mM NaCl), 2.5× PBS, 5× PBS, or 10× PBS for 1 h at room temperature with moderate agitation. For the last 10 min of labeling, PI was added to 1 μg/ml. Cells were pelleted, resus-

pended in 100 µl of the same buffer, and the fluorescence distribution of 10^6 events weres analyzed at ~1500 s^{-1} on the FACSort. The analysis of TNB–Cy5 conjugate was done on a MoFlo (Cytomation, Fort Collins, CO).

Phage panning and FACS sorting of phage libraries. The Griffin.1 library was rescued and subjected to five rounds of panning essentially according to the website instruction manual (http://www.mrc-cpe.cam.ac.uk/phage/g1p.html). Immunotubes were coated with 10 µg/ml conjugate overnight at 4°C in 50 mM carbonate buffer pH 9.3. Following each pan, the neutralized triethylamine eluates were halved and used to infect either TG-1 for the next round or ABLEC for FACS analysis. Eluate titers were monitored to indicate enrichment of antigen-binding phage.

For phage library sorting, the cells were grown in terrific broth, induced with 0.1 mM IPTG, and labeled with 100 nM probe in 5× PBS. Sorting was performed in exclusion mode on 10^6 events (10^7 for round 2) at no more than 1,000 events/s. The collected sort solution was passed through 0.2 µm membrane filters (Millipore Corporation, Bedford, MA), and cells withheld by the filters were allowed to grow after placement on SOC agar plus appropriate antibiotics at 30°C for 24 h.

Analysis of FECS-isolated clones from phage libraries. Small-scale fractionation and western blotting was performed according to Hayhurst and Harris[27]. A 10 µl aliquot from a 1 ml fraction derived from 20 ml OD units/cm at A_{600} was electrophoresed on a 12% Laemmli gel using the Bio-Rad (Hercules, CA) mini-protean cell. Samples were electroblotted to Immobilon P (Millipore) and probed with rabbit anti-β-lactamase or anti-APH-II sera (5 prime-3 prime, Boulder, CO) followed by anti-rabbit horseradish peroxidase (HRP) conjugate (Bio-Rad). ScFv proteins were detected using mouse anti-His6 (Sigma, St. Louis, MO) followed by anti-mouse HRP (Bio-Rad). Detection was carried out using SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL). ELISA was done on 100 µl of periplasmic fraction, or a 100 µl aliquot of phage supernatant using nonfat dried milk as a blocking agent and anti-his6/anti-mouse HRP for detecting scFv and anti-M13-HRP (Amersham Pharmacia) for detecting phage. ELISA points represent the average of duplicate wells. The entire fractionation and ELISA process was carried out three times on separate occasions and, while the absolute values varied slightly, the trend was always as shown herein.

Periplasmic expression and purification of scFvs. We used 40 ml terrific broth cultures of clones of interest to produce scFv for biophysical characterization. Arabinose or *lac* promoter–based vectors were induced with either 0.2% L-arabinose or 0.1 mM IPTG, respectively. Expression was allowed to continue for 4 h at 25°C. Protein was purified from osmotic shock fluid using immobilized metal affinity chromatography followed by size exclusion chromatography (Superdex-75, Pharmacia) to isolate monomeric scFv. Protein concentration was determined by A_{280} using an extinction coefficient of 44,850 or bicinchoninic acid assay using BSA as a standard.

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Pronunciation: di-'fyüz
Function: *verb*
Inflected Form(s): **dif·fused**; **dif·fus·ing**
Etymology: Middle English *diffused,* pp., from Latin *diffusus,* past participle
transitive senses
1 a : to pour out and permit or cause to spread freely **b :** EXTEND, SCATTER **c :** to spread thinly or wastefully
2 : to subject to diffusion; *especially* **:** to break up and distribute (incident light) by reflection
intransitive senses
1 : to spread out or become transmitted especially by contact
2 : to undergo diffusion
- dif·fus·ible /di-'fyü-z&-b&l/ *adjective*

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Definition

diffuse [Show phonetics]
verb [I or T]
1 to (cause something to) spread in many directions:
Television is a powerful means of diffusing knowledge.

2 to (cause a gas or liquid to) spread through or into a surrounding substance by mixing with it:
*Oxygen diffuses from the lungs **into** the bloodstream.*
The drop of red dye diffused slowly in the water.

diffuse [Show phonetics]
adjective
1 spread out and not directed in one place:
a diffuse light
The company has become large and diffuse.

2 DISAPPROVING not clear or easy to understand:
a diffuse literary style

diffusely [Show phonetics]
adverb

diffuser, diffusor [Show phonetics]
noun [C]
a device which is used to make light less direct, especially one used with a fluorescent light

diffusion [Show phonetics]
noun [U]
the process of diffusion in gases/liquids/solids

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